Small cantilevers for force spectroscopy of single molecules

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We have used a simple process to fabricate small rectangular cantilevers out of silicon nitride. They have lengths of 9–50 μm, widths of 3–5 μm, and thicknesses of 86 and 102 nm. We have added metallic reflector pads to some of the cantilever ends to maximize reflectivity while minimizing sensitivity to temperature changes. We have characterized small cantilevers through their thermal spectra and show that they can measure smaller forces than larger cantilevers with the same spring constant because they have lower coefficients of viscous damping. Finally, we show that small cantilevers can be used for experiments requiring large measurement bandwidths, and have used them to unfold single titin molecules over an order of magnitude faster than previously reported with conventional cantilevers. © *1999 American Institute of Physics.* [S0021-8979(99)04916-6]

I. INTRODUCTION

Since its invention by Binnig, Quate, and Gerber in 1986, atomic force microscopy (AFM) has proven to be an excellent tool for imaging a wide class of systems such as semiconductors, minerals, polymers, and biomaterials.[1,2] More recently, the AFM has been used to do single molecule force spectroscopy on a wide range of molecules.[3–6] Force spectroscopy can provide structural information about macromolecules, as well as information about the energy landscape of molecular bonds.[7] Increasing the dynamic range and sensitivity of the AFM in force spectroscopy experiments will provide a more useful tool for probing single molecules.

In the AFM, forces are measured by monitoring the deflection of a flexible cantilever. In the earliest AFMs, cantilevers were fashioned from millimeter sized hand-cut aluminum strips. Today micromachining techniques are used to mass produce well defined cantilevers with dimensions on the order of 100 μm.[8,9] Decreasing cantilever dimensions to the order of microns gives much higher resonant frequencies than larger cantilevers (>500 kHz in air), while simultaneously providing the same spring constants[10–12] (<100 mN/m). Therefore small cantilevers should allow for faster measurements. Furthermore, recent work by Gittes and Schmidt[13] has shown that, in principle, it is possible to lower the minimum detectable force a cantilever can measure by decreasing the cantilever's coefficient of viscous damping. Because smaller cantilevers have lower coefficients of viscous damping than their larger counterparts, they should be able to measure smaller forces.

We have used a simple process to fabricate arrays of small rectangular cantilevers out of low stress silicon nitride. The cantilevers are 9–50 μm long, 3–5 μm wide, and 86 and 102 nm thick. We have characterized these cantilevers through their thermal spectra in both air and water, and demonstrate that smaller cantilevers can measure smaller forces than larger cantilevers with the same spring constant. We also show small cantilevers can be used for experiments requiring a greater measurement bandwidth, and have used them to unfold single titin molecules with pulling speeds over an order of magnitude faster than previously performed with conventional cantilevers.

II. CANTILEVER FABRICATION

Typical materials used for micromachining cantilevers are silicon and silicon nitride. However, these materials have low reflectivity, making them a poor choice for optical detection schemes such as optical beam deflection, because low light levels at the detector can decrease the signal to noise ratio (SNR) of the measurement. Thus, many commercially available cantilevers made from silicon or silicon nitride are coated with metals such as gold or aluminum to increase reflectivity. Unfortunately, this has the undesirable effect of creating a temperature sensitive ''bimaterial'' strip that can give rise to spurious signals due to small changes in room temperature. This effect becomes especially large for smaller cantilevers where the thickness of the metal layer becomes comparable to the cantilever thickness.[14] A possible alternative is to make cantilevers solely out of metal. However, as we have reported elsewhere, it is difficult to control the stress in thin metal films that causes most metal cantilevers to bend.[15] One way to avoid these problems is to add a metallic reflector pad to the cantilever end in order to maximize the reflectivity while minimizing sensitivity to temperature changes.[16]

We fabricated small silicon nitride cantilevers both with and without gold reflector pads at their ends. In practice we found that the SNR of force measurements made with the cantilevers without pads (spring constants <100 mN/m) is

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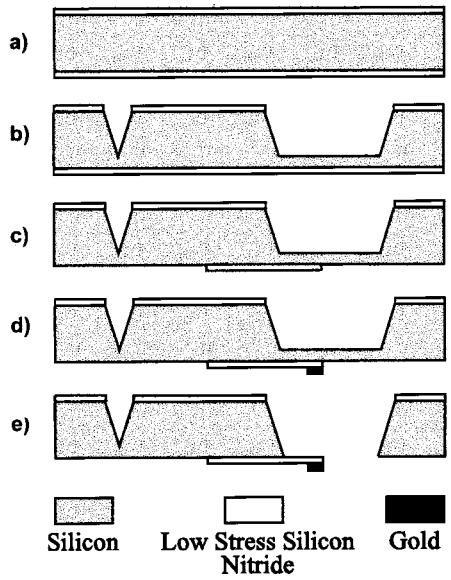

FIG. 1. Schematic of the three-mask process used to fabricate small silicon nitride cantilevers with gold reflector pads. The reflector pads serve to maximize reflectivity and minimize transmission while minimizing cantilever sensitivity to temperature changes.

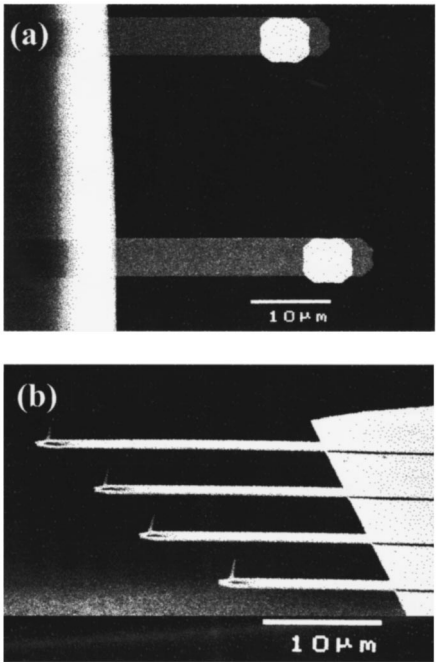

FIG. 2. (a) A SEM micrograph showing the top view of two small rectangular cantilevers with integrated reflector pads. The cantilevers are 5 μm wide, 86 nm thick, and 27 and 32 μm long. The pads are nominally 100 nm thick. (b) A SEM micrograph showing a perspective view of an array of cantilevers without pads. The tips were grown by electron beam deposition. The cantilevers are 5 μm wide, 102 nm thick, and 14–24 μm long.

limited by the thermal motion of the cantilevers themselves and not by detection noise. Therefore the addition of metal pads to increase reflectivity (and signal) does not increase the sensitivity of these cantilevers.[17] However, another problem that arises with nonreflective cantilevers is that light can pass through the cantilever and reflect off the sample back to the detector, where it interferes with light reflected from the cantilever itself. This interference causes a periodic signal that is dependent on the cantilever-sample separation[18] and can be much larger than signals caused by real deflections of the cantilever. The cantilevers with pads at their ends reduced light transmission through the cantilever and therefore minimized interference effects without significantly increasing cantilever sensitivity to temperature changes.

The process we used to fabricate small cantilevers is outlined in Fig. 1. First, a thin layer of low stress silicon nitride is deposited by low-pressure chemical vapor deposition onto a double sided polished, 300-μm-thick, $\langle 100 \rangle$ oriented single crystal silicon wafer [Fig. 1(a)]. Next, a deep etch pattern is defined in the silicon nitride layer on the noncantilever side of the wafer via photolithography and reactive ion etching. This pattern serves to define an array of chips held into a frame with a single silicon tab at the back of each chip. Next, KOH is used to anisotropically etch through the silicon wafer until a thickness of about 10–15 μm remains as shown in Fig. 1(b). Then the cantilever pattern is defined in the silicon nitride by reactive ion etching [Fig. 1(c)] and 3–5 nm of chrome followed by 20–100 nm of gold is evaporated onto the cantilever side of the wafer and patterned into pads at the ends of the cantilevers [Fig. 1(d)]. Next, 20–60 nm of plasma-enhanced-chemical-vapor-deposited silicon nitride is deposited on the cantilever side of the wafer to passivate the exposed silicon. Finally, the cantilevers are released by etching the remaining 10–15 μm of silicon in KOH and by removing the plasma-enhanced-chemical-vapor-deposited silicon nitride with buffered HF[19] as shown in Fig. 1(e).

Figure 2(a) is a scanning electron microscopy (SEM) micrograph showing the top view of two cantilevers made via this process. These cantilevers are nominally 5 μm wide, 86 nm thick, and 27 and 32 μm long. The gold pads visible at the cantilever ends are nominally 100 nm thick. Although the gold pads are slightly off center, this did not cause any problems while using the cantilevers. Figure 2(b) is a micrograph showing a perspective view of an array of cantilevers without pads. The tips were grown by electron beam deposition.[20] The cantilevers are nominally 5 μm wide, 102 nm thick, and 14–24 μm long.

III. CHARACTERIZING SMALL CANTILEVERS

To characterize the cantilevers we measured their thermal deflection spectra with a prototype AFM that employs an optical beam detection system. The prototype AFM was designed to be used with small cantilevers by employing high numerical aperture optics to achieve a focused spot size of 1.6 μm in diameter.[12,21] Measurements were taken at room temperature in both air and water. For spectra taken in air, the simple harmonic oscillator amplitude response function $A(\nu)$ was fit to the data

$$A(\nu) = A_{\mathrm{dc}} G(\nu), \tag{1}$$

where

$$G(\nu)=\frac{\nu_0^2}{\sqrt{(\nu_0^2-\nu^2)^2+\dfrac{\nu_0^2\nu^2}{Q^2}}} \tag{2}$$

and where ν_0 is the resonant frequency, Q is the quality factor, and A_{dc} is the cantilever amplitude at zero frequency. Specifically, only the data on the peak of the first mode of vibration are fit because these data are clearly dominated by the thermal motion of the cantilever and not by other noise sources. From the fits to the thermal spectra taken in air, we were able to calculate ν_0, Q, and A_{dc}. Then we used the equipartition theorem to calculate spring constants k in a method that has been described in detail previously.[10,22] Finally, we calculated the coefficient of viscous damping R by using the relation[23] $R=k/(2\pi\nu_0 Q)$. We also used the thermal spectra taken in water to characterize the cantilevers in a liquid environment. However, it has been shown that the analogy with a simple harmonic oscillator is only appropriate in the limit of small dissipative effects, or more specifically when the quality factor Q is much greater than one.[24] Although this condition was usually satisfied for cantilevers in air, cantilevers in water had quality factors of order one. Therefore, the simple harmonic oscillator response function can only provide an estimate of the resonant frequencies for cantilevers in liquid.

Figure 3(a) shows the measured resonant frequencies in both air and water[25] plotted as a function of length for an array of cantilevers without pads which are 15–50 μm long, 5 μm wide, and 86 nm thick. The fit to the expected $(\text{length})^{-2}$ dependence is also plotted.[26] Figure 3(b) shows the measured spring constants for the same set of cantilevers as well as the fit to the expected $(\text{length})^{-3}$ dependence.[26] Finally, Fig. 3(c) shows the coefficient of viscous damping for these same cantilevers as measured in air.

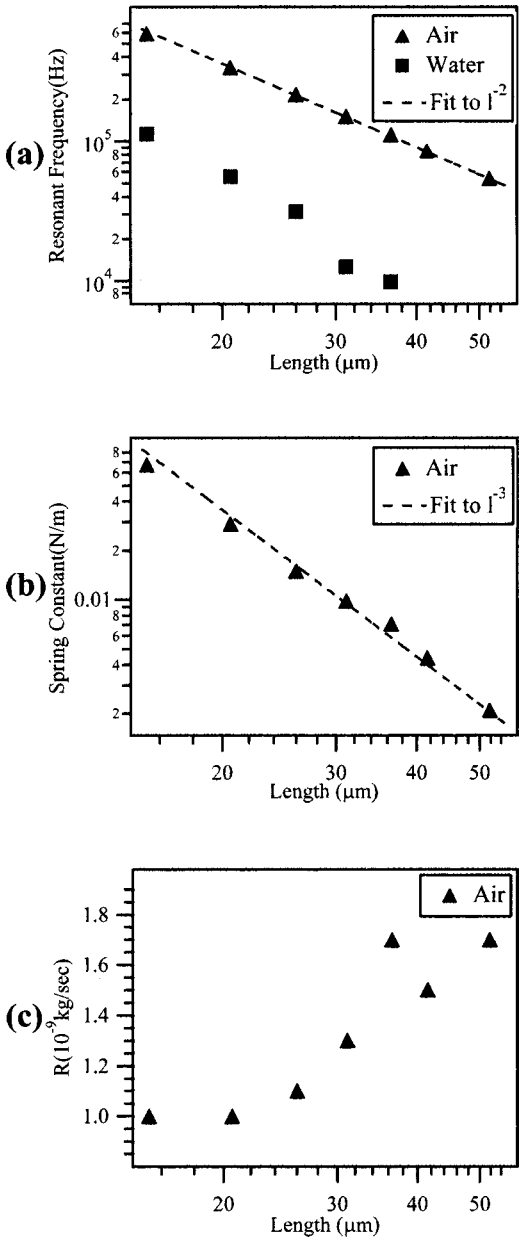

FIG. 3. (a) Measured resonant frequencies in air and water plotted as a function of length for an array of rectangular cantilevers which are 5 μm wide and 86 nm thick. The resonant frequencies in air are fit to the expected $(\text{length})^{-2}$ dependence. (b) Measured spring constants for the same cantilevers are plotted along with the fit, which predicts a $(\text{length})^{-3}$ dependence. (c) The coefficients of viscous damping R as measured in air.

IV. MINIMUM DETECTABLE FORCE

The smallest force an AFM can measure at room temperature is limited by both noise from the detection system used to monitor the cantilever motion and by thermal motion of the cantilever itself. In general, the SNR of a measurement made with bandwidth B of an oscillating force with amplitude $F(\nu)$ will have the form[26]

$$\text{SNR}=\frac{\dfrac{F(\nu)}{k}G(\nu)}{\sqrt{\dfrac{4k_BTRB}{k^2}G^2(\nu)+(\text{Detector Noise})^2}}, \tag{3}$$

where k_B is Boltzmann's constant and T is the temperature of the measurement. The first term in the denominator is thermal noise as described by the fluctuation-dissipation theorem, which requires any system that has energy dissipation R to also experience fluctuations.[27] In general, the energy dissipation R will be system dependent and can be caused by many different mechanisms such as viscous damping, acoustic reradiation, or magnetic eddy-current damping.[28] In the case of a simple harmonic oscillator, viscous damping causes all energy dissipation; therefore R is equal to the coefficient of viscous damping as defined previously.[29] The second term in the denominator is a generalized frequency dependent detector noise (with units of displacement), which has many contributions including photonic shot noise for optical detection schemes, amplifier noise, and $1/f$ noise.[26] In general, detection noise is independent of the spring constant of the cantilever, therefore the signal to noise ratio will be maximized by decreasing the spring constant k to a point where the thermal noise dominates the detector noise. By taking the

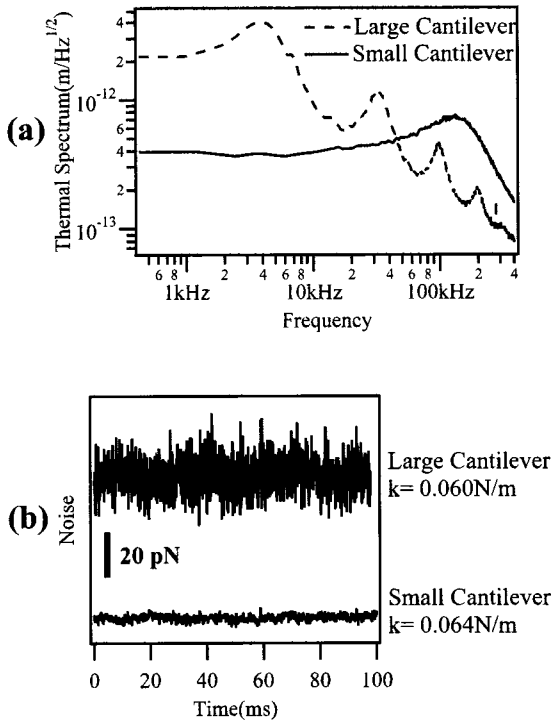

FIG. 4. (a) Thermal spectra of a large (200-μm-long) and small (10-μm-long) cantilever immersed in water far from the sample surface. (b) Measured force noise for the same two cantilevers in a 3 kHz bandwidth.

limit of Eq. (3) for small k and setting SNR=1 we get the Nyquist relation for the minimum detectable force of a thermally limited measurement

$$F_{\min} = \sqrt{4k_B TRB}. \tag{4}$$

As can be seen from Eq. (4), the only method for decreasing the minimum detectable force at a fixed bandwidth B is by either minimizing the coefficient of viscous damping R,[13] or by lowering the temperature of the experiment. However, in many experiments involving biological molecules the environment must be a room temperature buffer solution. Therefore, the only way to decrease the minimum detectable force for these experiments is to modify the cantilever dimensions so that the coefficient of viscous damping R is decreased.

In Fig. 3(c), the measured values of R are plotted as a function of cantilever length for an array of cantilevers in air. As might be expected, R decreases with decreasing cantilever size. Therefore, a direct consequence of Eq. (4) is that smaller cantilevers can measure smaller forces. For example, we found the values of R in air of typical commercially available silicon nitride cantilevers to be as much as 30 times larger than those of our small cantilevers having comparable spring constants and quality factors. This means that for thermally limited measurements, small cantilevers are able to detect forces more than five times smaller than large cantilevers.

Similar reductions in noise level are also achievable when using small cantilevers in liquid environments. In Fig. 4(a) the measured thermal spectra of a large cantilever and small cantilever in water are shown.[30] The large, commercially available cantilever[31] is 200 μm long and has a spring constant of 60 mN/m. The small cantilever is 10 μm long and has a similar spring constant of 64 mN/m. In order to determine the smallest low frequency force that these cantilevers can measure we immersed them in water and recorded the deflection signal. The deflection signal was low pass filtered at 3 kHz and then multiplied by the measured spring constant of the cantilever. Figure 4(b) shows the measured force in pN for both cantilevers. The standard deviation of the force gives a noise level of 7.4 pN for the large cantilever and 1.3 pN for the small cantilever. The noise level for both cantilevers is dominated by the cantilever's thermal motion (detector noise contributed to less than 5% of the total noise in both cases). Therefore, the improved sensitivity of the smaller cantilever is believed to be a direct consequence of a decrease in the coefficient of viscous damping R.

It is important to note that for actual force spectroscopy experiments, the minimum detectable force will be somewhat different than reported here for two reasons. First, the measurements presented here were taken with the cantilevers far from the sample surface. The noise level should increase for small separations as the coefficient of viscous damping increases from fluid squeezing effects.[23] Second, for cases where the effective spring constant of the probed molecule is comparable to the spring constant of the cantilever, effects of the molecule will play a role in the noise level.[13]

V. MEASUREMENT BANDWIDTH

In addition to being able to measure smaller forces, small cantilevers can also be used for force measurements with greater bandwidth. In general, a sinusoidal force with amplitude $F(\nu)$ applied to a cantilever with spring constant k will give a measured cantilever deflection

$$X(\nu) = \frac{F(\nu)}{k} G(\nu). \tag{5}$$

The form of $G(\nu)$ will depend on the details of the system. As discussed previously, for cantilevers immersed in air it will usually suffice to take $G(\nu)$ to be given by Eq. (2), whereas cantilevers immersed in liquid will have a more complicated response function. Therefore, it is necessary to know the amplitude response function of a cantilever in order to obtain a force from a measured cantilever deflection. If we assume the thermal noise spectrum of a cantilever to be proportional to the cantilever's amplitude response function,[32] then the bandwidth over which a cantilever can be used for force measurements can be evaluated. In Fig. 4(a), it can be seen that the response of the large cantilever begins to decrease at 4 kHz while the response of the small cantilever with similar spring constant remains flat up to 100 kHz. Consequently, small cantilevers should be useful for measuring forces over a much larger bandwidth than larger cantilevers having comparable spring constants.

In order to demonstrate the increased measurement bandwidth of small cantilevers, we used them to stretch single molecules of the protein titin more than an order of magnitude faster than previously performed with conventional cantilevers. Samples were prepared in a similar fashion to what has been described previously.[5] The cantilever

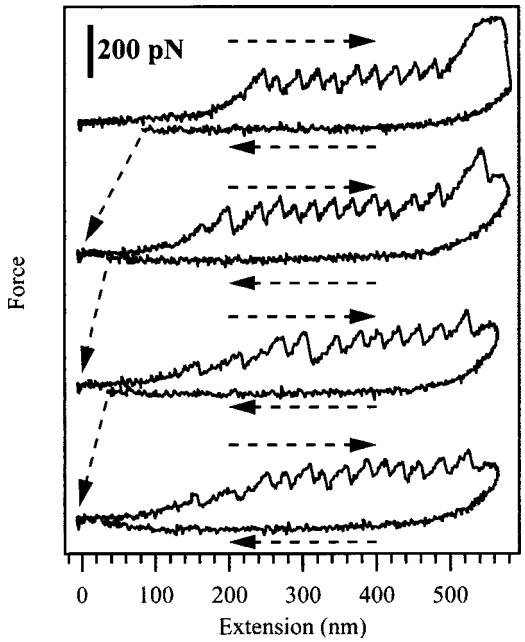

FIG. 5. (a) Four consecutive force spectra on a single molecule of the protein titin using a small cantilever. All force spectra were acquired at a pulling rate of 30–39 μm/s, which is an order of magnitude faster than previously performed with conventional cantilevers.

used was similar to those shown in Fig. 2(a) and had a spring constant of 23 mN/m and a resonant frequency of 23 kHz in water. In Fig. 5, the force versus extension curves are shown for four consecutive ''pulls'' of a single molecule of titin. The observed sawtooth pattern clearly shows the unfolding of individual protein domains as has been reported.[5] Between pulls we paused for 30 s to allow the protein domains to refold. The first two pulls were performed at 30 μm/s and the second two pulls were performed at 39 μm/s. These pulling speeds are an order of magnitude faster than previously performed and correspond to a frequency in the sawtooth pattern of 1.2 and 1.6 kHz.

VI. CONCLUSION

We have used a simple process to fabricate small rectangular cantilevers out of silicon nitride. We have measured the cantilever's resonant frequencies, spring constants, and coefficients of viscous damping and have shown that small cantilevers can measure smaller forces than larger cantilever with the same spring constant. Finally, we have used the increased resonant frequency of small cantilevers to unfold single titin molecules more than an order of magnitude faster than previously performed.

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[30] These spectra were obtained with the following conditions. The deflection signal was low pass filtered at 300 kHz and digitized at 1 MHz for the large cantilever. The deflection signal was low pass filtered at 1 MHz and digitized at 2.5 MHz for the small cantilever.
[31] The narrow 200-μm-long V-shaped silicon nitride cantilever sold by Digital Instruments, Santa Barbara, CA 93117.
[32] The assumption that the thermal spectrum is proportional to the cantilever response function is true if the thermal noise driving force is independent of frequency. Although we have not experimentally verified that the thermal noise driving force is independent of frequency, we are unaware of any measurements or theoretical prediction that would indicate this not being true for the bandwidth of interest here.